Detection of *EGFR* Gene Mutation in Lung Cancer by Mutant-Enriched Polymerase Chain Reaction Assay

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Abstract

Purpose: Mutations in the epidermal growth factor receptor (*EGFR*) gene have been reported to be present in non – small cell lung cancer (NSCLC) and related to the responsiveness of tumors to EGFR tyrosine kinase inhibitors, suggesting its usefulness as a biomarker. Because clinical samples contain tumor and normal cells or genes, a highly sensitive assay for detecting mutation is critical for clinical applications.

Experimental Design: The mutant-enriched PCR is a rapid and sensitive assay with selective restriction enzyme digestion. We developed the mutant-enriched PCR assay targeting exons 19 and 21 of *EGFR* and applied the developed assay to detect mutations in 108 cases of surgically resected specimens of NSCLCs, 18 samples of computed tomography (CT) – guided needle lung biopsies, and 20 samples of pleural fluid. In addition, results were then compared with those from direct sequencing and a nonenriched PCR assay.

Results: The mutant-enriched PCR that was proved to enrich one mutant of 2×10^3 normal genes detected mutations in 37 cases of 108 resected tumors, seven samples of CT-guided lung biopsies, and seven samples of pleural fluid. Among mutant cases, four resected tumors, two CT-guided lung biopsies, and two pleural fluid were identified as additional mutant cases by the mutant-enriched PCR, which were considered normal based on nonenriched assays.

Conclusions: Our results indicate that *EGFR* mutations are readily detectable by mutant-enriched PCR in various clinical samples. Thus, mutant-enriched PCR may provide a valuable method of potentially detecting a small fraction of mutant genes in heterogeneous specimens, indicating its possible use in clinical application for NSCLC.

Lung cancer is the leading cause of cancer deaths throughout the world, with over one million cases diagnosed every year (1). The prognosis of lung cancers has not reached a satisfactory level because of the absence of a generalized early detection system, the frequency of metastases at the time of diagnosis, recurrence after surgery, and poor responsiveness to chemotherapy. With understanding of the cancer-specific molecular features, new approaches based on novel knowledge have been attempted for the diagnosis and treatment of non – small cell lung cancer (NSCLC; refs. 2, 3). Recently, selective tyrosine kinase inhibitors of the epidermal growth factor receptor (EGFR) have been used to treat NSCLC, with responses observed in some populations (4). In addition, several more recent reports have shown that *EGFR* mutations are present in NSCLC, especially adenocarcinoma, and that clinical responsiveness to EGFR tyrosine kinase inhibitors was significantly associated with somatic mutations in the tyrosine kinase domain of the *EGFR* gene in NSCLCs (5 – 10). About 85% to 90% of these mutations occurred at exons 19 and 21 near the ATP cleft of the tyrosine kinase domain where 4-anilinoquinazoline compounds, such as gefitinib, compete with ATP for binding (5 – 11). Therefore, *EGFR* mutations at exons 19 and 21 may be useful molecular marker for treatment of NSCLC.

Clinical samples, such as the specimen by biopsy, pleural fluid, sputum, stool, and plasma, may contain a small fraction of mutated genes and a large amount of wild-type genes. Detecting genetic alterations in these specimens using a sensitive assay has been reported to be useful for early detection and monitoring of disease recurrence (12 – 15). Recent advancements in molecular technology have enabled the design of sensitive detection assays for genetic alterations. Among them, the mutant-enriched PCR is a rapid assay with a high specificity and sensitivity that can detect one mutant gene among as many as 10^3 to 10^4 copies of the wild-type gene (16 – 18). Thus, establishment of a mutant-enriched PCR assay to detect *EGFR* mutations would be very useful for the management of patients with NSCLCs.

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The purpose of this study was (*a*) to develop a convenient and sensitive PCR-based assay for mutations in *EGFR* exons 19 and 21 and (*b*) to apply the developed assay for detecting *EGFR* mutations in clinical samples, including specimens by biopsies, pleural fluid, and surgically resected tissues from patients with NSCLC.

Materials and Methods

Construction of plasmids containing exons 19 and 21 of the* EGFR *gene. pLN-R3.0 plasmids containing *EGFR* exon 19 wild type, deletion type (delE746-A750), exon 21 wild-type, and point mutant (L858R) type, derived from human primary NSCLC and nonmalignant lung tissue, were used to validate the mutant-enriched PCR assay for *EGFR* mutation in exons 19 and 21. The sequences of each plasmid were confirmed by sequencing.

Mutant-enriched PCR analysis for* EGFR *gene mutation in exon 19. Mutant-enriched PCR is a two-step PCR with intermittent restriction digestion to eliminate wild-type genes selectively, thus enriching the mutated genes. The sequences of primers for PCR amplification are as follows: 5′-ATCCCAGAAGGTGAGAAAGATAAAATTC-3′ (forward primer, ex19-S1) and 5′-CCTGAGGTTCAGAGCCATGGA-3′ (reverse primer, ex19-AS1). In this assay, the first round of amplification was done for 17 cycles for resected specimens or 22 cycles for other samples (20 seconds at 94°C, 30 seconds at 60°C, 20 seconds at 72°C) using 5 to 100 ng of sample DNA, 150 μmol/L deoxynucleotide triphosphate, 2 pmol of each primer, and 0.25 units of HotStarTaq DNA polymerase (Qiagen, Inc., Valencia, CA). The principle behind the assay is shown in Fig. 1A. *Mse*I was used to digest the TTAA sequence (from first letter of codon 747 to first letter of codon 748) in wild-type genes, which is frequently absent in exon 19 deletion mutants (codons 747-749; Leu-Arg-Glu sequence), resulting in the enrichment of deletion-type genes. Because the TTAA sequence was also present at 26 bases upstream from the first letter of codon 747, a forward mismatch primer was designed to create a 1-base mismatched (ATAA, T to A) sequence at this site, preventing *Mse*I digestion at the primer attaching site in the amplicon. Intermittent restriction digestion of the 2 μL of the first PCR products was done using 10 IU of *Mse*I at 37°C for 4 hours. In addition, an aliquot was used as a template for the second round of PCR amplification under the same conditions as the first round PCR but for 40 cycles. The product of the second amplification was analyzed on 12% PAGE via sliver staining. Because the range of exon 19 deletions containing commonly deleted codons 747 to 749 was from 9 to 18 bp in our pervious report (10), differences in the sizes of the PCR amplicons enabled us to distinguish mutant from wild-type amplicons.

Mutant-enriched PCR analysis for* EGFR *gene mutation in exon 21. The mutant-enriched PCR analysis for exon 21 was basically the same as that for exon 19 except for the primer sequences and restriction enzyme (Fig. 1). The sequences of the primers for PCR are as follows: 5′-CAGCCAGGAACGTACTGGTGA-3′ (forward primer, ex21-S1), 5′-CGCAGCATGTCAAGATCACAGAT-3′ (forward primer, ex21-S2), and 5′-TCCCTGGTGTCAGGAAAATGCT-3′ (reverse primer, ex21-AS1). The restriction enzyme *Msc*I was used to digest (TGGCCA) sequence in the amplicon of the wild-type gene. In contrast, mutant type (L858R) was not digested because of the base substitution of T to G at first base of TGGCCA, resulting in the enrichment of the L858R mutant. The conditions and reagent constitution for PCR amplification were the same as those used for exon 19. The first round of PCR amplification using these primers was done using ex21-S1 and ex21-AS1 primers. After digestion using *Msc*I, the second PCR was done using a different forward primer (ex21-S2), designed for inside of the first PCR amplicon (Fig. 1B). The second PCR product was digested with *Sau*96I (GGNCC) for RFLP analysis using electrophoresis on 12% PAGE via sliver staining. As described above, because *Msc*I digests the wild-type (CTG), incomplete digestion could be misinterpreted as a false-positive result; thus, complete digestion was required when *Msc*I was used as a restriction enzyme for RFLP analysis. By contrast, *Sau*96I digests the mutant type of codon 858(CGG), and wild type is not digested, showing that even partial digestion of the PCR product indicates the presence of a mutation. This is the reason why *Sau*96I was used to digest second PCR products for RFLP analysis.

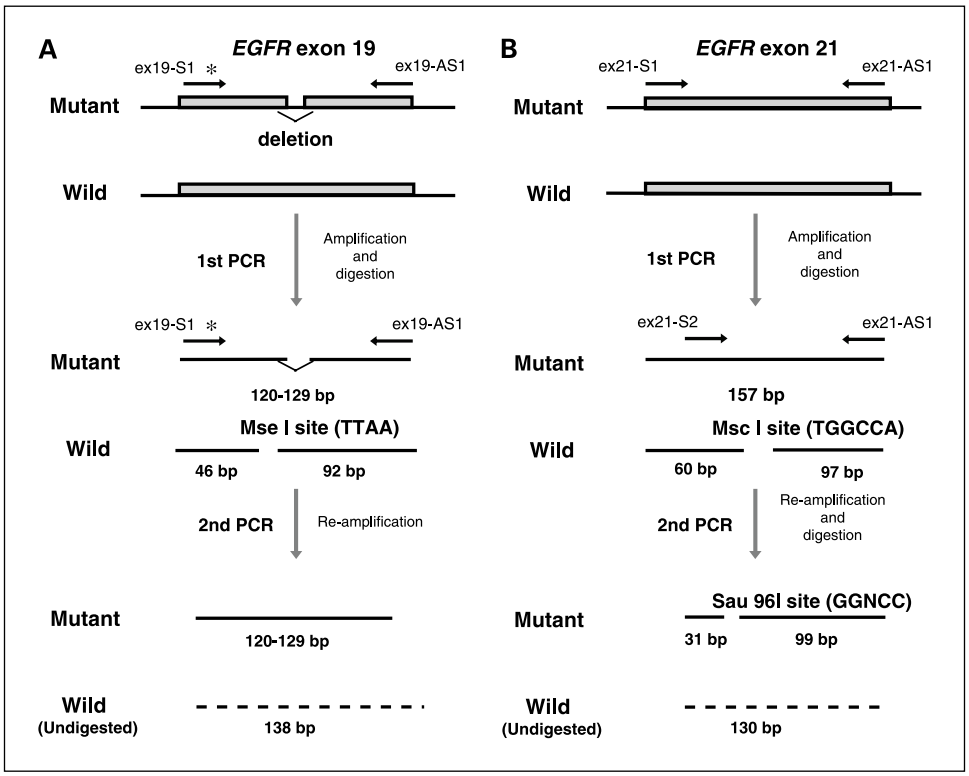

Fig. 1. Principle of mutant-enriched PCR assay for exons 19 and 21 of the *EGFR* gene. *A,* mutant-enriched PCR assay detecting deletion of exon 19. First round PCR was done using ex19-S1 and ex19-AS1 primers. To enrich PCR amplicons containing exon 19 deletion, the first PCR product was digested with *Mse*I resulting in digestion of only wild type. The second PCR amplification was done with same primers, and amplified products were analyzed by PAGE. *B,* mutant-enriched PCR assay detecting mutation of L858R. First round PCR was done using ex21-S1 and ex21-AS1 primers. After the digestion with *Msc*I, reamplification was done with ex21-S2 and ex21-AS1 primers and digested with *Sau*96I. The box shows the exons of the *EGFR* gene, and the arrows denote primer sites. *, the 1-base mismatched site (ATAA) in the ex19-S1 primer.

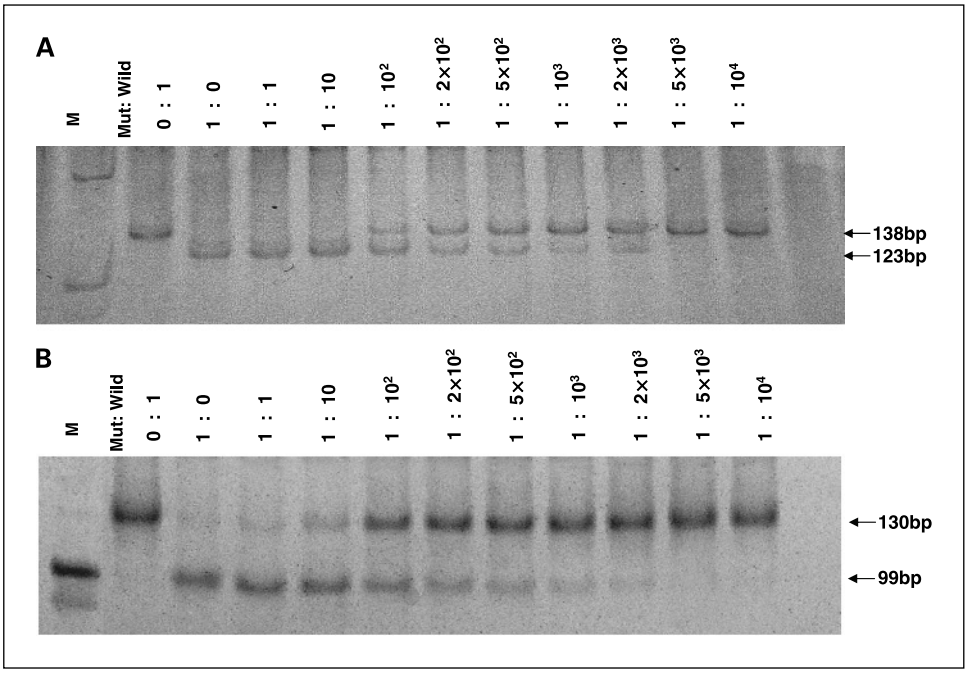

Fig. 2. Evaluation of sensitivity of mutant-enriched PCR for *EGFR* exons 19 and 21. The pLN-R3.0 plasmid, which contained *EGFR* mutant of exon 19 or 21, was mixed with wild type contained plasmid to dilute mutant *EGFR* from 1- to 10^4-fold. *A,* The bands of 123 bp indicate mutant alleles, and bands of 138 bp indicate wild-type alleles. The mutant allele at the 123-bp band was detected up to the level of 2×10^3 -fold dilution. *B,* the bands of 99 bp indicate digested mutant alleles, and bands of 130 bp indicate wild-type alleles. The mutant allele at the 99-bp band was detected up to the level of 2×10^3-fold dilution. M, DNA marker; Mut, control DNA that contains mutant allele; Wild, wild type.

Nonenriched PCR assays for* EGFR *gene exons 19 and 21. We also analyzed samples using nonenriched PCR-based assays. The same sets of primers were used for the PCR reactions of exon 19 (ex19-S1 and ex19-AS1) and exon 21 (ex21-S2 and ex21-AS1), done for 40 cycles, respectively. The common deletions of exon 19 were distinguished from the wild type based on PCR product length polymorphisms detected using 12% PAGE. For exon 21, PCR-RFLP with *Sau*96I digestion followed by electrophoresis was done. These PCR-based assays and direct sequencing were regarded as "nonenriched assays."

The mutant-enriched or nonenriched PCR assays for all mutant cases and 40 randomly selected wild-type cases of primary tumors were done at least twice and the same results were obtained.

EGFR *gene sequencing.* *EGFR* mutations in the extracted DNA were examined using PCR-based direct sequencing for exons 19 and 21. To confirm the results of the mutant-enriched PCR assay, the second PCR products were directly sequenced for both exons 19 and 21; in addition, DNAs were isolated from the gels, purified, and sequenced for exon 19 assay. Sequencing was done using Applied Biosystems PRISM dye terminator cycle sequencing method (Perkin-Elmer Corp., Foster City, CA) with ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) in Central Research Laboratory, Okayama University Medical School.

Clinical samples and DNA extraction. DNA from 108 cases of surgically resected frozen specimens that had been previously examined in our study (72 cases of adenocarcinomas, 33 squamous cell carcinomas, two adenosquamous carcinomas, and one large cell carcinoma) by direct sequencing (10) were analyzed by mutant-enriched and nonenriched PCR assays. In addition, 18 samples of computed tomography (CT)–guided needle lung biopsies from primary tumors and 20 samples of pleural fluid from patients with recurrent or advanced NSCLCs were examined with the same assays. Pleural fluid was collected from pleural effusion after the removal of cells. The permission of the institutional review board and informed consent from each patient were obtained. DNA was isolated from the tissue samples by digestion with proteinase K followed by phenol/chloroform (1:1) extraction and ethanol precipitation in the 108 frozen specimens and by DEXPAT (TaKaRa, Shiga, Japan) in the 18 samples of CT-guided biopsies embedded in paraffin according to the kit manufacturer's instructions. Free DNA in 1 mL from each of the 20 pleural fluid samples was extracted using the QIAmp DNA blood kit (Qiagen), according to the manufacturer's protocol for blood and body fluid. As nonmalignant samples, 30 nonmalignant peripheral lung tissue specimens' corresponding tumors and five pleural fluid samples from patients with tuberculosis were used for analysis. The concentration of DNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE).

Results

Sensitivity of mutant-enriched PCR for exons 19 and 21 of the* EGFR *gene. To evaluate the sensitivity of the mutant-enriched PCR assays, we used serially diluted plasmid DNAs containing *EGFR* mutant and wild-type genes. Although the mutant-enriched PCR assays for exon 19 deletions and exon 21 point mutations in *EGFR* genes failed to detect one mutant of 5×10^3 wild-type genes, they were able to detect one mutant in the presence of 2×10^3 wild-type genes (Fig. 2). On the other hand, the nonenriched PCR assays for exons 19 and 21 detected one mutant of 10 wild-type genes but not of 20 wild-type genes. Thus, we concluded that the nonenriched PCR assays for exons 19 and 21 could detect one mutant of 10 wild-type genes.

EGFR *gene mutations in clinical samples.* We examined 108 specimens of primary NSCLCs using mutant-enriched and nonenriched PCR assays and compared the results with those obtained by direct sequencing in our previous study (10). The results of the direct sequencing showed that 16 of the 108 (15%) tumors exhibited exon 19 deletions and 17 (16%) exhibited exon 21 point mutations (L858R). The results of the nonenriched PCR and direct sequencing analyses were consistent. With the mutant-enriched PCR assay, however, we identified one additional mutant case in exon 19 and three cases in exon 21 that had not been detected by direct sequencing (Table 1). Among these four cases, two cases had received neoadjuvant chemoradiation therapy because of N_2 disease. The pathologic findings for one of the resected specimens revealed very few viable cells. This fact may explain why the direct sequencing and nonenriched assay did not

Table 1. The summary of *EGFR* mutations by nonenriched and enriched PCR assays

	EGFR exon 19 mutation		*EGFR* exon 21 mutation	
	Nonenriched (%)	Enriched (%)	Nonenriched (%)	Enriched (%)
Lung cancer				
Resected tumor (n = 108)	16 (15)	17 (16)	17 (16)	20 (19)
CT-guided biopsy (n = 18)	4 (22)	4 (22)	1 (6)	3 (17)
Pleural fluid (n = 20)	3 (15)	5 (25)	2 (10)	2 (10)
Nonmalignant specimen				
Peripheral lung (n = 30)	0	0	0	0
Pleural fluid (n = 5)	0	0	0	0

NOTE: n, no. sample. Nonenriched, direct sequencing and nonenriched PCR assay. Enriched, mutant-enriched PCR assay. CT-guided biopsy, CT-guided needle lung biopsy.

detect the mutation, but the mutant-enriched PCR was able to detect the mutation, probably from a small fraction of tumor cells. Of interest, the pathologic examination of transbronchial lung biopsy and mediastinal lymph node specimens of this case were diagnosed as the central type of squamous cell carcinoma arising in a female never smoker. Direct sequencing of this lymph node specimen revealed an L858R mutation that was also detected in the transbronchial lung biopsy and surgically resected specimens.

We also examined 18 specimens obtained by CT-guided needle lung biopsies and 20 samples of pleural fluid. The examples of PCR-based assays are shown in Fig. 3, and the results are summarized in Tables 1 and 2. Table 2 showed only mutant cases in CT-guided biopsy and pleural fluid samples with nonenriched or enriched PCR assay. In specimens by CT-guided biopsy, exon 19 deletions were detected in 4 of the 18 samples by both direct sequencing and the nonenriched PCR assay. Mutant-enriched PCR for exon 19 detected the same deletions but did not detect any deletions in any additional samples. Of note, as shown in PAGE (Fig. 3A and B), the mutant-enriched PCR assay selectively amplified mutant alleles of sample C14, whereas nonenriched PCR for C14 showed the presence of both mutant and wild-type bands in PAGE analysis. As for exon 21, although the nonenriched assays revealed only one mutation, the mutant-enriched assays identified two additional mutant cases (C5 and C16), which were confirmed

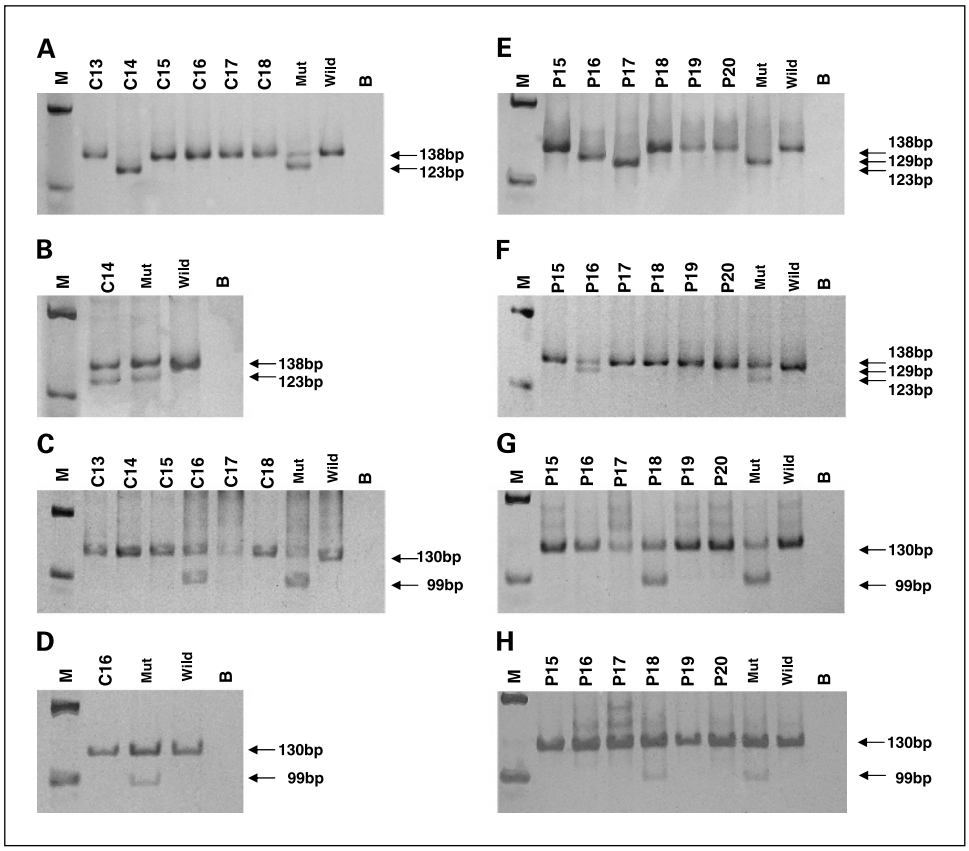

Fig. 3. Results of mutant-enriched PCR for the CT-guided biopsy and pleural fluid. The result of mutant-enriched PCR assay (*A*) and nonenriched PCR assay (*B*) of exon 19 and mutant-enriched PCR assay (*C*) and nonenriched PCR assay (*D*) of exon 21 in CT-guided needle lung biopsies. Both mutant-enriched and nonenriched PCR assays for exon 19 detected mutant alleles of sample C14, but mutant-enriched PCR assay selectively amplified mutant alleles of this case. Nonenriched PCR assay for exon 21 could not detect mutant allele of C16, whereas mutant-enriched PCR assay detected mutation in this case. The result of mutant-enriched PCR assay (*E*) and nonenriched PCR assay (*F*) of exon 19 and mutant-enriched PCR assay (*G*) and nonenriched PCR assay (*H*) of exon 21 of pleural fluid samples. Mutant-enriched PCR assay for exon 19 showed mutant in P17, which was shown to be wild type by nonenriched PCR assay. M, DNA marker; Mut, control DNA that contains mutant allele; Wild, wild-type; B, water blank.

Table 2. Comparison between nonenriched and enriched PCR in clinical samples

No.	Pathology	Nonenriched	Enriched	Mutational type
C3	Ad	Exon 19	Exon 19	delE746-A750
C5	Ad	w	Exon 21	L858R
C6	Ad	Exon 19	Exon 19	delE746-A750
C8	Ad	Exon 21	Exon 21	L858R
C11	Ad	Exon 19	Exon 19	delE746-A750
C14	Ad	Exon 19	Exon 19	delE746-A750
C16	Ad	w	Exon 21	L858R
P1	Ad	w	Exon 19	delL747-T751insP
P5	Ad	Exon 19	Exon 19	delE746-A750
P11	Ad	Exon 21	Exon 21	L858R
P13	Ad	Exon 19	Exon 19	delE746-A750
P16	Ad	Exon 19	Exon 19	delL747-A750 insP
P17	Ad	w	Exon 19	delE746-A750
P18	Ad	Exon 21	Exon 21	L858R

NOTE: Nonenriched, nonenriched PCR assay. Enriched, mutant-enriched PCR assay.
Abbreviations: Ad, adenocarcinoma; w, wild type; C, CT-guided biopsy sample; P, pleural fluid sample.

by sequencing (Table 2). As an example of the CT-guided needle lung biopsy, Fig. 4 shows the pathologic findings for "C16," in which a small fraction of cancer cells were present in a large excess of nonmalignant cells. Only the mutant-enriched PCR assay successfully detected the exon 21 mutation in this case. Regarding the pleural fluid samples, three and two cases were identified as bearing exon 19 deletions and exon 21 mutations, respectively, using nonenriched assays. With the mutant-enriched assay, two additional mutant case (samples P1 and P17) in exon 19 were identified (Table 2). The corresponding surgically resected specimens were included among the 108 cases of primary NSCLCs examined, and the mutational pattern of primary tumors and CT-guided biopsies were identical. The corresponding resected tumor of the pleural fluid sample was available in one case, and it exhibited the same type of mutation as that seen in the corresponding pleural fluid.

As nonmalignant samples, we examined 30 peripheral lung tissue specimens and five pleural fluid samples from patients with tuberculosis and found no mutations in any of the samples using both mutant-enriched PCR and nonenriched assays. These results indicated the high specificity of the mutant-enriched PCR assay for *EGFR* exons 19 and 21.

Discussion

In this study, we successfully established a PCR-based assay for screening mutations at frequently affected sites of *EGFR*, enabling us to analyze clinical samples rapidly and accurately. Needless to say, a highly sensitive assay is critical for the detection of cancer-derived mutant *EGFR* genes among a great excess of wild-type genes. In addition, the frequent *EGFR* amplification in NSCLC can be helpful for detecting *EGFR* mutants (19), because the presence of amplified mutant genes should increase the possibility of detection. Indeed, our mutant-enriched PCR assay detected *EGFR* alterations that were not identified with a nonenriched assay, especially in CT-guided biopsies and pleural fluid specimens. Thus, our results strongly suggested that a highly sensitive assay is necessary to detect mutations in these clinical samples.

The detection of mutations in pleural fluid specimens also shows that tumor DNA may be released into pleural fluid through the apoptosis or necrosis of disseminated tumor cells, and that *EGFR* mutations were detectable in pleural fluid from these patients. Cytologic examination of pleural effusion is a rapid, efficient, and minimally invasive method of diagnosing cancer but is not routinely warranted for light microscopy alone

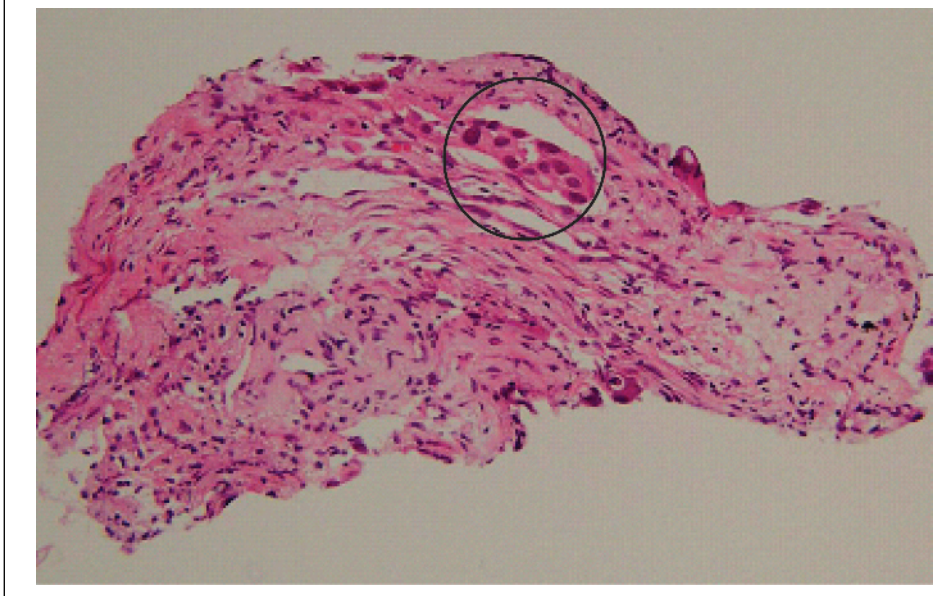

Fig. 4. Microscopic finding of the specimen obtained by CT-guided needle lung biopsy. The portion circled by black line revealed a small fraction of tumor cells in the specimen.

(sensitivity, 40-87%; ref. 20). Taking together these facts, the genetic analysis of pleural fluid, even after the collection of cells in the sample, may be useful for diagnosis and determining treatment strategies. Dai et al. showed that the cytologic analysis of pleural effusion was applicable for the molecular detection of tumor recurrence using *p53* and *K-ras* gene mutations as effective biomarkers (13). With primary tumors, the use of mutant-enriched PCR did not dramatically increase the number of *EGFR* mutations that were detected. This fact indicates that the *EGFR* mutation may be present in the majority of cancer cells in the specimen, suggesting that such mutations may be an early event in the multistep tumorigenesis leading to NSCLCs.

Regarding the mutant-enriched PCR assay, two issues should be noted for. (*a*) The restriction site of wild-type fragments may be destroyed by the erroneous replacement of a critical nucleotide, mainly because of the high PCR cycle number, leading to a false-positive result (13). For this reason, we confirmed our results with the mutant-enriched PCR assay at least twice and also did sequencing to confirm the *EGFR* alterations. Because no discrepancies were found, the assay conditions were considered to be appropriate. (*b*) The developed mutant-enriched PCR assay can only be used to analyze specific alterations containing a commonly deleted region of exon 19 (codons 747-749) and the L858R mutation of exon 21. Thus, this assay cannot detect minor alterations, like mutations in exon 18, minor deletions of exon 19, and exon 20 insertions. New assays that can sensitively detect all types of *EGFR* mutations should be developed in the future for the accurate screening of clinical samples from NSCLC patients.

In summary, we developed a highly sensitive assay for detecting alterations in exons 19 and 21 of *EGFR* and showed that *EGFR* mutations can be detected in clinical samples. The mutant-enriched PCR assay for detecting *EGFR* mutations in various kinds of clinical samples may provide a valuable method for potentially detecting and monitoring recurrences of NSCLC and for predicting tumor responsiveness to *EGFR* tyrosine kinase inhibitors.

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